The Role of CXCR3 in Breast Cancer Tumorigenesis in a CXCR3 Over-expressed Murine Model

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Introduction

According to the nonprofit organization Breastcancer.org, approximately one in eight women develop breast cancer in their lifetime and breast cancer in U.S. women has the second highest mortality rate of all cancers, second only to lung cancer. The immune system is critical in the bodies response against cancer, with the chemokine receptor CXCR3 known to play a role in various cancers, including breast cancer. CXCR3 is expressed by a variety of immune cells, including but not limited to natural killer cells (NK), natural killer T cells (NKT), CD4+ and CD8+ T cells, and T regulatory cells (Treg). [1; 2; 3]. CXCR3 has also been shown to be expressed on macrophages and CXCL10 has been shown to play a role in monocyte chemotaxis *in vitro* [4]. These CXCR3 expressing cells migrate to specific areas of the human body through following chemokine gradients made up of three different ligands. The ligands associated with CXCR3 are CXCL9, CXCL10, and CXCL11 [1].

Previous research has shown the role of CXCR3 within other cancers, such as its role in promotion of tumor growth in gliomas [5]. This research has also shown that Natural killer T cells (NKT), which are known to express CXCR3, have been found around and infiltrating gliomas in higher levels than controls [5]. Other cell types that are known to express CXCR3 have also been examined in their role against breast cancer, like natural killer cells which have previously been shown to play a role in suppression of tumor growth and metastasis [6]. Previous work has also helped characterize the leukocyte response in the tumor tissue, axillary lymph nodes, and peripheral blood of breast cancer patients [7]. However, even though the role played by immune cell types that express CXCR3 has been examined in breast cancer, the actual role of

CXCR3 within tumorigenesis in breast cancer has not been extensively examined. Of the published works, a large focus is on metastasis from the angle of tumor cells expressing CXCR3 [8]; however, little has been published on the role of CXCR3 in the host response to breast cancer tumorigenesis. Instead of relying on an *in vivo* model to elucidate the role of CXCR3 within breast cancer, the literature focuses on the use of clinical samples, which do not allow for the accountability of environmental, genetic, or other factors [9].

Due to the lack of a host response focus in previous research, this project's focus is on the importance of CXCR3 in response to tumorigenesis from the host perspective through the use of an animal model. We hypothesize that CXCR3 is essential for tumor suppression due to its importance in the accumulation of immune cells at the site of tumorigenesis. Therefore, it is expected that the over-expression of CXCR3 will lead to the development of smaller tumors than in the wild type animal.

Methods

This project used transgenic mice that have been genetically modified to over-express the gene for CXCR3 in a C57Bl/6 background. These mice have been created by Dr. Oghumu as part of his work in Dr. Satoskar's laboratory. These transgenic mice were injected with 500,000 cells in 100 uL of PBS per mouse of a cancerous cell line to induce tumors within the mammary gland tissue. Wild type mice were also injected with the same cell line to act as a control for the transgenic mice. The cell line being injected has a modified gene that encodes the Polyoma middle T oncoprotein (PyMT) inserted into its genome and are provided by Dr. Ganju's laboratory. The PyMT gene is an oncogene and is engineered to have the Murine Mammary Tumor Virus (MMTV) promoter. This MMTV promoter allows the expression of the oncogene

to be mammary gland specific, thereby allowing greater specificity in the type of cancer generated by injecting the cell line. This cell line has been shown to develop a cancer that is very similar to human breast cancer in development and cellular morphology, thereby proving to be a good model for the immune response to tumorigenesis [10].

After injection of the cancerous cell line, the tumor growth was monitored weekly. The tumor growth was measured with a caliper to determine the width and length of the tumor. The smaller measurement was designated as the width and the formula $0.52 * \text{length} * (\text{width})^2$ was used to determine the volume. Once the tumor had grown to between 1.5 and 2 cm in length and width, the mice were sacrificed and analysis of the tumor and surrounding tissue was undertaken. This group of mice ended up being sacrificed seven weeks after the injection of the cell line.

The sacrificed animals were then dissected to extract the tumor and surrounding tissue for analysis. The lymph nodes and spleen were also collected to analyze the draining and migration of cells that occurs as part of the natural immune process. Analysis of these samples was conducted by flow cytometry.

In flow cytometry, cells are exposed to antibodies that bind to specific antigens, and these antibodies are then detected through florescence. This florescence stems from the excitation of the fluorochrome on the ends of the antibodies by lasers within the flow cytometry machine, thereby producing detectable florescence. This technique was used to detect the lymphocyte and macrophage populations. CD3 in junction with CD4 and CD8 were used as markers to determine the CD8+ (CD3+ and CD8+) and CD4+ T (CD3+ and CD8+) cells within the samples. The NK marker NK1.1 and the T cell receptor marker CD3 were the antibodies used to determine the NK (CD3- and NK1.1+) and NKT (CD3+ and NK1.1+) cell populations. Classically activated and alternatively activated macrophages were also examined. The classically activated macrophages

were determined through staining with antibodies against the mature macrophage markers F4/80 and CD11b. The alternatively activated macrophages populations were determined through antibodies against the mannose receptor (CD206), which is an alternative macrophage marker due to its up-regulation after IL-4 activation of the macrophage.

A repeat of this initial experiment has been started with slight modifications to the experimental design. The second experiment contains seven Tg and 12 WT mice, rather than the initial experiment's five mice per group. Also, 1,000,000 PyMT cells in 100 uL of PBS were injected per mouse rather than the initial experiments 500,000 cells per mouse.

Results

The initial signs of tumor growth were detected at one and a half weeks, with the ability to feel a minuscule bump around the ninth nipple of three of the WT and two of the Tg mice (Figure 1). By the second week these tumors became measurable with the WT mice averaging a tumor volume of 8.9 across three mice that had measurable tumors and two mice with unmeasurable tumors. At this two week time point the Tg mice averaged 2.2 across two mice with measurable tumors and two mice with unmeasurable tumors. At the third week time point, the mice displayed some superficially noticeable tumor regression. The Tg mouse group had four mice with

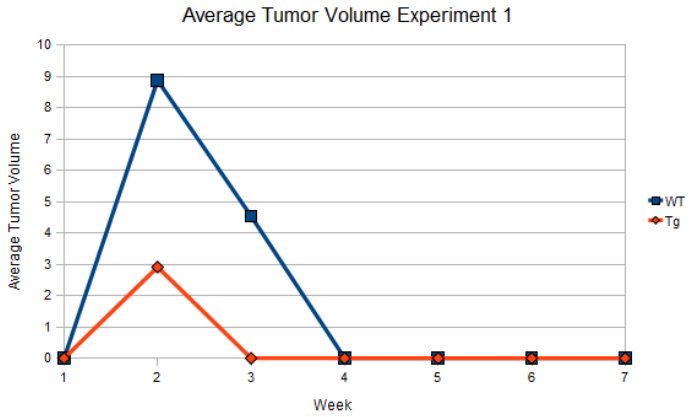

Figure 1

unmeasurable but detectable tumors at three weeks, while the wild type also had four mice with unmeasurable tumors and a fifth mouse with a measurable tumor. The fourth week post-injection continued the trend of the regression of the initial tumor growth, with all five WT mice having

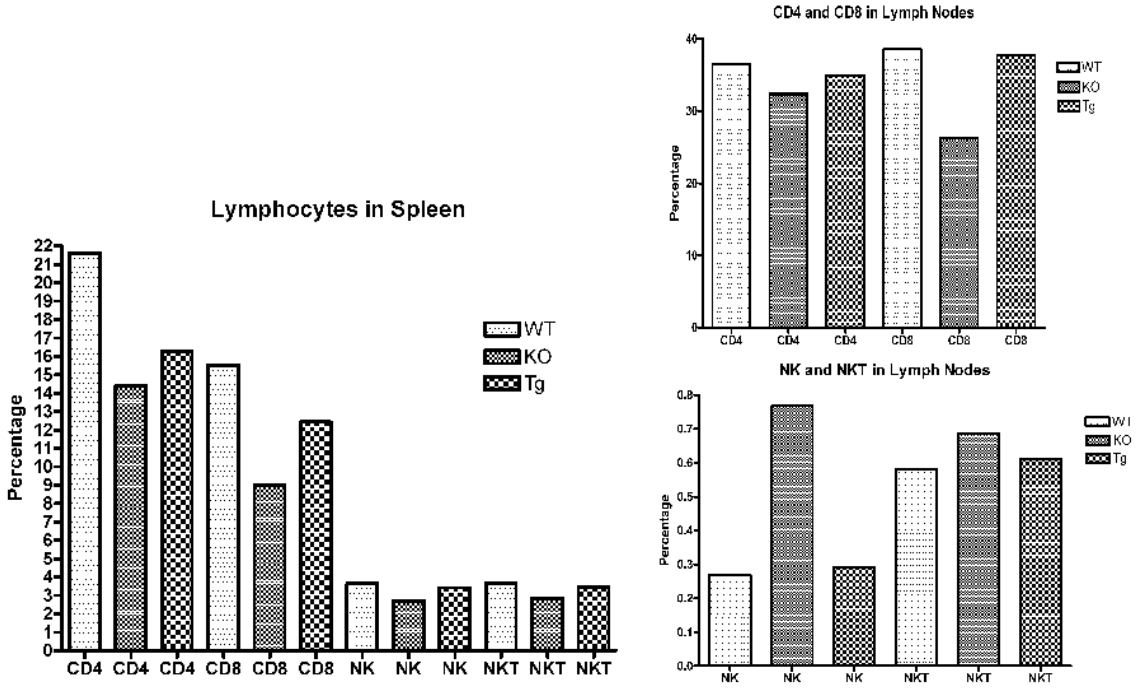

Figure 2

unmeasurable tumors and four of the Tg mice also having unmeasurable tumors. After the fourth week neither of groups had mice with measurable tumors (Figure 1). These mice were not sacrificed until the seventh time point, because a similar experiment that utilized CXCR3 knockout mice that was being run in-tandem with this experiment. The knockout mice had a tumor that grew to the tumor size limit seven weeks post-injection, and the mice from both experiments were then sacrificed simultaneously.

Flow cytometry was used to more closely examine the differences in immune cell trafficking and populations within the WT and Tg mice, in response to tumorigenesis. Within the lymph node, the CD4+ and CD8+ T cell percentages of the total cells in the lymph node were similar between the WT and Tg mice (Figure 2). The percentage of NK and NKT cells that made up the lymph node population was also consistent between the WT and Tg mice groups. This consistency between NK and NKT levels in the two groups was also seen in the mouse spleens. However, the WT mice had a higher percentage of CD4+ T cells and CD8+ T cells in the spleen than the Tg mouse group. Flow cytometry was also used to examine the macrophage populations in the two groups of mice. In the lymph node, the Tg mouse group had a higher percentage of alternatively activated macrophages than the WT mice. Even though the spleen contained a significantly larger percentage of alternative macrophages than the lymph node, the relation between the alternative macrophage percentages of the WT and Tg mice groups has remained the same. Within the spleen, the Tg mice had a higher percentage of the M2 macrophages than the WT mouse group (Figure 3).

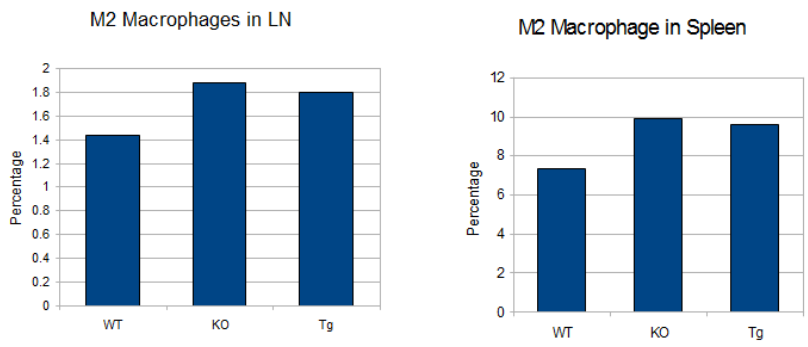

Figure 3

This experiment was repeated with minor changes to the experimental methods, and is currently ongoing. This second experiment has already yielded results in the form of tumor volume size up to 40 days post-injection of the PyMT cell line. The Tg and WT mouse groups are exhibiting very similar average tumor volumes up to the 28th day. After this point, the average tumor volume is significantly larger in the Tg mouse group than in the WT mice (Figure 4). More results will be available from this second experiment upon sacrificing the animals and harvesting their organs for further analysis.

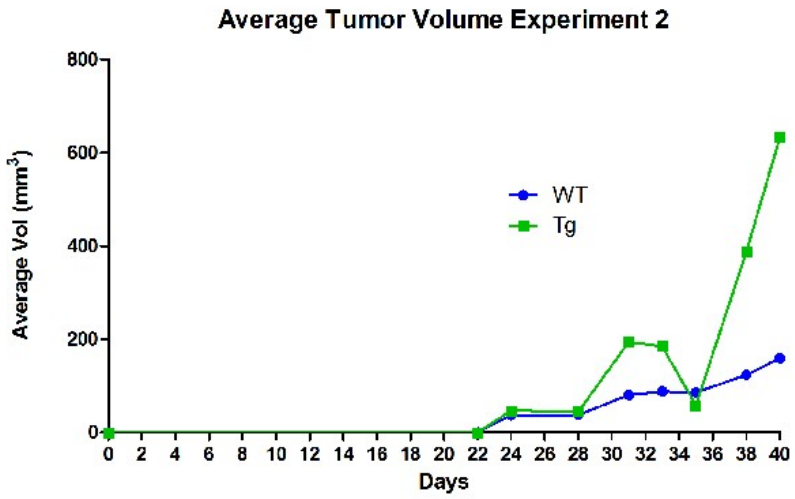

Figure 4

Discussion

The results from the first experiment suggest a role of CXCR3 within the immune response to tumorigenesis. While the difference between the tumor growth in the Tg and WT groups is noticeable, thereby suggesting CXCR3 is playing a role in the response to tumor growth, the difference is not a very large one. However, the WT mice did have significantly higher T lymphocyte percentages in the spleen, pointing towards an effect on T cell trafficking

by CXCR3 during tumorigenesis. Interestingly, a higher percentage of alternatively activated macrophages was found in both the spleen and the lymph nodes of the Tg mice combined. This raised level of macrophages and the knowledge that macrophages express CXCR3 further supports the idea of a change in immune cell trafficking within the Tg mice [4].

Combined with the immune cell population data, the tumor volumes after the 28 day time point in the second experiment may indicate an effect on tumorigenesis and breast cancer development stemming from the difference in cell trafficking due to CXCR3 expression. If a similar increase in alternative macrophages in the Tg mice is found in the second experiment as in the first experiment, then it could explain the significantly larger average tumor volumes in the Tg mice after the fourth week time point (28 day time point). Alternative macrophages are known to be immune suppressive and it has been shown that tumor-associated macrophages are typically alternatively activated macrophages [4; 11]. This observation would then point to increased immune regulatory cells trafficking to the tumor and existence in the periphery due to the over-expression of CXCR3. The higher trafficking of immune suppressor cells would also help explain the lower levels of CD4+ T cells and CD8+ T cells within the Tg mice spleens in comparison to the WT mice spleens.

Now that it is evident that CXCR3 over-expression is having a noticeable effect on tumorigenesis, it is going to be important to continue experiment two and design and execute more experiments to better elucidate how CXCR3 is causing this change in tumorigenesis. Due to the possibility of immune regulatory cells playing a role in this change, it will be important to examine the populations of T regulatory cells in the lymph node, spleen, and tumor. Flow cytometry will also need to be done on the tumor itself, to better understand the effect of cell trafficking on the immune cell population in and around the tumor. It may also be interesting to

harvest lymph nodes individually to examine whether there is a difference in the immune cell populations between lymph nodes local to the tumor versus sites far away from the tumor. While further experiments are definitely needed to gain a better idea of the role of CXCR3 in breast cancer tumorigenesis, this work has been a promising start with great potential.

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